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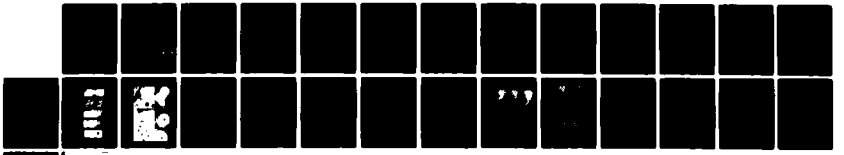

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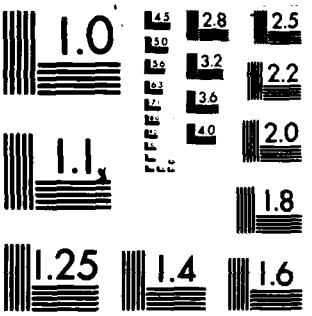

GENETIC AND MOLECULAR STUDIES OF THE PHLEBOTOMUS FEVER GROUP OF VIRUSES

ANNUAL REPORT

DR. DAVID H.L. BISHOP
AUGUST 1980

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

During the reporting period we have continued studies which have led to characterizing the structural components of several Phlebotomus fever serogroup viruses (viral polypeptides and, or RNA species). The viruses studied include Punta Toro (PT), Karimabad (KAR), Chagres (CHG), Sandfly fever Sicilian (SFS Tesh and Sabin isolates). Sandfly fever Naples (SFN), Rift Valley fever (RVF), Phlebotomus 3 virus (Phl 3), Icoraci (ICO) and Buenaventura (BUE) viruses. In summary, the evidence has shown that the viruses have 3 virion RNA

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species ~~(large 2.6-2.8x10^6, medium 1.85-2.2x10^6 and 0.7-0.8x10^6)~~, which are comparable in number to those of other members of the family, but different in size for the S RNA ~~(which is significantly larger than the S RNA of Bunyavirus genus members i.e. 0.4-0.5x10^6)~~. The studies have also shown that, although the N polypeptides of Phlebotomus fever group viruses ~~(20-24x10^3 daltons)~~ are similar in size to those of bunyaviruses, the glycoproteins ~~(57-69x10^3 daltons)~~ are quite different ~~(bunyaviruses: G1 115x10^3, and G2 38x10^3 daltons)~~. Thus both the RNA and polypeptide analyses have shown that the members of serogroup are distinct from members of the Bunyavirus genus. This evidence, developed for the Phlebotomus fever serogroup viruses, plus the serological data and evidence developed from bunyaviruses and nairoviruses has been the basis for the proposal of the formation of new genera of viruses in the family Bunyaviridae (Phlebovirus and Nairovirus genera). Publications relating to this information and supported by the contract are given in the Bibliography of the Progress Report (Bishop, 1980; Bishop et al., 1980; Cash et al., 1980; Clerx and Bishop, 1980; Clewley and Bishop, 1980; Ushijima et al., 1980)

We have found it necessary to modify the standard genetic recombination test to demonstrate genetic recombination btween ts mutants of PT virus. By pairwise crosses three nonoverlapping genetic recombination groups of PT ts mutants have been defined. Group I has 8 ts mutants; Group II has 5 ts mutants; Group III has 1 ts mutant (so strictly is not a group); 1 ts mutant is probably a double mutant.

Competition RIA assays have been undertaken using iodinated nucleocapsid and glycoprotein preparations of KAR virus, KAR antisera and the competing antigens of KAR, CHG and SFS. The results obtained indicate that the KAR and SFS N polypeptides have more antigenic determinants in common than have the N polypeptides of KAR and CHG. Also the KAR and SFS G polypeptides share more antigenic determinants than the G polypeptides of KAR and CHG. No shared antigenic determinants were detected between KAR and vesicular stomatitis virus (VSV), La Crosse (LAC), Oriboca (ORI) or Bunyamwera (BUN) viral antigens.

Icoraci virus has been adapted to produce plaques in Vero cell monolayers at 39.8°C. The original virus stock, which gave 10^5 plaques at 35°C, gave none at 39.8°C. By high temperature passaging of the virus stock and cloning at 39.8°C, a stock of ICO virus has been derived which gives $8x10^4$ PFU at 35°C and $2.3x10^3$ PFU at 39.8°C. Further adaptation is being undertaken with this and other phleboviruses in order to set the stage for heterologous virus genetic recombination experiments.

Fingerprinting analyses have been undertaken at USAMRIID on RVF virus isolates obtained from Egypt, Uganda, Rhodesia and South Africa.

I. SUMMARY

The reporting period represents the last 12 of the 30 months since the inception of the project. During the previous 18 months reporting period we: (1) characterized several of the major structural components of the Phlebotomus fever group viruses (Robeson, et al, 1979), (2) isolated a few temperature sensitive (ts), conditional lethal, mutants of Punta Toro (PT) virus, and (3) demonstrated the feasibility of using oligonucleotide fingerprinting to distinguish Dengue (DEN) virus serotypes using cloned Aedes albopictus cells to produce labeled virus (these procedures have now been adopted by WRAIR personnel for their Dengue virus studies). The results of the prior reporting period were as follows:

(1). Analyses of the major structural components of the Phlebotomus fever viruses established that Karimabad (KAR), PT, Chagres (CHG), Candiru (CDU), Itaporanga (ITP) and the Sicilian and Naples sandfly fever (SFS, SFN) viruses, each has a tripartite RNA genome and three major structural polypeptides (two glycoproteins, G1 and G2, and an internal, nucleocapsid associated, protein N). Both the mol. wt. of the major structural polypeptides and the virion RNA segments of the different Phlebotomus fever viruses were easily distinguished from those of the California serogroup viruses and to various extents (depending on the virus) from each other. Tryptic peptide analyses of ^{35}S and ^{3}H methionine labeled G1 and G2 polypeptides of KAR established that these two polypeptides have distinguishable sequences. The behaviour of reduced KAR G1 and G2 polypeptides on polyacrylamide gel electrophoresis was found to be aberrant by comparison with unreduced preparations.

(2). Twenty four ts mutants of PT virus were isolated following mutagenesis of the wild-type virus by growth in the presence of 5-fluorouracil. Recombination assays with five of these mutants were performed without detecting recombination.

(3). Oligonucleotide fingerprint analyses of prototype DEN 1, 2, 3 and 4 viruses involving both single and mixed coelectropherograms of ribonuclease T1 digests of ^{32}P labeled 40S viral RNA samples showed that each has a unique fingerprint that is easily distinguished from that of another prototype DEN virus. Evidence was obtained indicating that the 5' sequence of DEN 2 RNA is m7GpppAmpXp...

In the current reporting period we have made the following progress relevant to the aims of the contract. We have: (4) demonstrated high frequency genetic recombination between 15 ts mutants of PT virus and have defined 3 non-overlapping genetic recombination groups; (5) undertaken structural and electron microscopic analyses on various phleboviruses; (6) analyzed, by radioimmune assays (RIA), the antigenic relationships of KAR, CHG and SFS glycoproteins and N proteins; (7) initiated genetic analyses on Icoraci (ICO) virus; and (8) at the request of USAMRIID, analyzed the genotypes of certain Rift Valley fever (RVF) virus isolates by fingerprinting. The results of the current reported period were therefore as follows:

(4). We have found it necessary to modify the standard genetic recombination test to demonstrate genetic recombination btween ts mutants of PT virus. By pairwise crosses three nonoverlapping genetic recombination groups of PT ts mutants have been defined. Group I has 8 ts mutants; Group II has 5 ts mutants; Group III has 1 ts mutant (so strictly is not a group); 1 ts mutant is probably a double mutant.

(5). Structural analyses on PHL group viruses are being continued in relation to characterizing the virion RNA species, polypeptides and surface structure arrangments.

(6). Competition RIA assays have been undertaken using iodinated nucleocapsid and glycoprotein preparations of KAR virus, KAR antisera and the competing antigens of KAR, CHG and SFS. The results obtained indicate that the KAR and SFS N polypeptides have more antigenic determinants in common than have the N polypeptides of KAR and CHG. Also the KAR and SFS G polypeptides share more antigenic determinants than the G polypeptides of KAR and CHG. No shared antigenic determinants were detected between KAR and vesicular stomatitis virus (VSV), La Crosse (LAC), Oriboca (ORI) or Bunyamwera (BUN) viral antigens.

(7). Icoraci virus has been adapted to produce plaques in Vero cell monolayers at 39.8°C. The original virus stock, which gave 10^5 plaques at 35°C, gave none at 39.8°C. By high temperature passaging of the virus stock and cloning at 39.8°C, a stock of ICO virus has been derived which gives 8×10^4 PFU at 35°C and 2.3×10^3 PFU at 39.8°C. Further adaptation is being undertaken with this and other phleboviruses in order to set the stage for heterologous virus genetic recombination experiments.

(8). Fingerprinting analyses have been undertaken at USAMRIID on RVF virus isolates obtained from Egypt, Uganda, Rhodesia and South Africa.

II. REPORT

A. Introduction.

The objectives of this contract are to determine the genetic capacity of the Phlebotomus fever group viruses and the implications of forming new Phlebotomus fever virus genotypes. Since members of this group of exotic viruses cause illnesses in epidemic proportions in different parts of the world, and therefore are of military significance, our objectives relate to the question of deriving vaccine strains of viruses which will be useful in protecting military and civilian personnel against virus infections.

To realize these objectives we aim to develop genetic tools in the form of temperature sensitive (ts), conditional lethal, mutants of particular member viruses, and use these in mixed virus infections to produce new virus genotypes by RNA segment reassortment (Gentsch & Bishop, 1976; Gentsch, et al., 1977b). By knowing the RNA segment coding assignments (Gentsch & Bishop, 1978, 1979), we will be able to produce custom genotypes of certain Phlebotomus fever group viruses containing particular genetic information and gene products. Such genotypes will eventually be tested for their pathogenicity and vaccine capabilities in model animal systems and compared to the prototype strains. If effective, then similar procedures will be used to derive reassortant viruses in clean cell systems in order to obtain virus vaccines that can be used to immunize man.

This report therefore describes both analyses of the genetic potential of selected Phelbotomus fever group viruses, and molecular studies to characterize the viral genomic RNA species and their gene products. The report only covers the items 4-8 listed in the Summary. It will not detail the results given in previous reports.

At the request of WRAIR, we have employed one of the biochemical procedures commonly used to differentiate virus isolates (namely oligonucleotide fingerprinting) to determine the feasibility of differentiating RVF viruses. The results of these studies are also given in this report.

4) Genetic studies with Punta Toro virus

The initial stock of PT virus gave plaques at 35°C but not at 39.8°C. After adaptation to growth at 39.8°C in Vero cell monolayers, plaques at 39.8°C were obtained. The plaque assay (McCown et al., 1979) had to be modified by decreasing the DMSO concentration in the overlay to 0.8% to reproducibly yield 2-3mm plaques at 39.8°C by 7 days post-infection, and 2-3 mm plaques at 35°C by 5-6 days post-infection. As reported previously some 24 ts mutants of PT virus have been selected from 500 progeny virus obtained in mutagenized virus stocks. In this reporting period we have determined that several of the ts mutants grew to low titer at 35°C and on repassaging to obtain higher titers, they either leaked badly (i.e. gave substantial numbers of plaques at 39.8°C) or reverted to a wild-type phenotype, and so were not good candidates for genetic studies. Shown in Table I are the 17 ts mutants that have reasonably low EOP values and high enough titers for genetic recombination studies. Two of the mutants (ts 10 and ts 15) have been held aside from the recombination experiments reported below because we will have to use alternate protocols for their analyses since their titers are in the low 10^6 category.

TABLE I

Titers and plating efficiencies of representative PT ts mutants

PT ts mutant	Titer 35°	Titer 39.8°C	EOP
1	8.0×10^7	7.3×10^5	9.1×10^{-3}
2	2.3×10^7	3.0×10^3	1.3×10^{-4}
3	1.0×10^8	1.0×10^5	1.0×10^{-3}
5	3.0×10^7	4.0×10^3	1.3×10^{-4}
6	2.5×10^7	2.0×10^4	8.0×10^{-4}
7	5.3×10^7	1.0×10^5	1.9×10^{-3}
8	1.4×10^7	1.0×10^3	7.0×10^{-5}
9	1.8×10^7	5.0×10^4	2.7×10^{-3}
*10	2.0×10^6	1.0×10^3	5.0×10^{-4}
13	3.3×10^7	1.0×10^3	3.0×10^{-5}
14	3.7×10^7	2.0×10^4	5.4×10^{-4}
*15	1.2×10^6	1.0×10^3	8.3×10^{-4}
16	3.0×10^7	1.0×10^3	3.3×10^{-5}
18	2.6×10^7	1.4×10^4	5.3×10^{-4}
23	1.9×10^7	1.0×10^4	5.0×10^{-4}
27	5.0×10^7	2.0×10^4	4.0×10^{-4}
29	7.0×10^6	6.0×10^4	8.5×10^{-3}
wt	6.5×10^7	5.0×10^7	7.6×10^{-1}

Each mutant virus stock was titered by plaque assays at 35°C (permissive temperature) and 39.8°C (non-permissive temperature). EOP values for each virus were calculated from their titer at 39.8°C divided by their titer at 35°C. *Low titers of these mutant virus stocks made them unsuitable for recombination assays.

Initial genetic recombination studies with a few ts mutants in pairwise crosses (see last year's report) were negative. Since this was not expected we repeated the study using the same and alternate mutants in pairwise crosses (ts 1, ts 2, ts 3, ts 5, ts 6, ts 7, ts 8 and ts 9). The data also indicated no recombination on direct assays of the virus in the supernatant fluids, but clear evidence for recombination when the infected cells were freeze-thawed to release cell associated virus (Table 2). Recombination values (%R) were calculated from the formula:

$$\%R = \frac{((AB_{35})_{39.8} - (A_{35})_{39.8} - (B_{35})_{39.8}) \times 100 \times 2}{(AB_{35})_{35}}$$

Where $(AB_{35})_{39.8}$ and $(AB_{35})_{35}$ represent the mixed ts virus infection grown at 35°C and assayed at 39.8°C and 35°C respectively, $(A_{35})_{39.8}$ and $(B_{35})_{39.8}$ are the single A or B ts mutant virus infections grown at 35°C and assayed at 39.8°C. The figure of 2 is included to represent the number of double ts reassortants that are expected to occur at frequencies equivalent in number to the wild-type reassortants. Whether they occur has not been proven.

TABLE 2

Recombination analyses of PT ts mutants

PT ts mutant	% Recombination with PT ts mutant[a]						
	2	3	5	6	7	8	9
ts 1	17	0	0	17	12	0	0
ts 2		27	5	0	0	29	32
ts 3			0	15	7	0	0
ts 5				9	2	0	0
ts 6					0	17	21
ts 7						2	14
ts 8							0

[a]%Recombination values were determined as described in the text.

Assignments: Group I: ts 1, ts 3, ts 5, ts 8, ts 9

Group II: ts 2, ts 6, ts 7

The ts mutants were categorized into groups (Group I, Group II) on the basis of their genetic recombination. Five mutants were assigned to Group I (ts 1, ts 3, ts 5, ts 8 and ts 9). Three were assigned to Group II (ts 2, ts 6 and ts 7). Mutants assigned to a group did not recombine with other mutants assigned to that group, however they did recombine with ts mutants assigned to the other group.

Two PT ts mutants assigned to Group I (PT I-1 and PT I-8) and two PT ts mutants assigned to Group II (PT II-2 and PT II-6) were used in recombination assays to screen seven other PT ts mutants (ts 13, ts 14, ts 16, ts 18, ts 23, ts 27 and ts 29). The results are presented in Table 3. From the results obtained three of the seven mutants were Group I mutants (ts 16, ts 27, ts 29), two Group II mutants (ts 13, ts 14), one (ts 18) recombined with both the representative Group I and Group II mutants and so can be considered as a Group III mutant, and one (ts 23) did not recombine with either the Group I or Group II viruses and probably represents a double mutant.

TABLE 3

Recombination analyses with representative ts mutants of PT virus

PT ts mutant	% Recombination with PT ts mutant[a]						
	13	14	16	18	23	27	29
ts 1	1	2	0	2	0	0	0
ts 2	0	0	10	3	0	4	5
ts 6	0	0	8	3	0	5	5
ts 8	5	9	0	6	0	0	0

[a]%Recombination values were determined as described in the text.

Assignments: (see table 2)
Group I: ts 1, ts 3, ts 5, ts 8, ts 9, ts 16, ts 27, ts, 29
Group II: ts 2, ts 6, ts 7, ts 13, ts 14
Group III: ts 18

Unassigned: ts 23

(5). Biochemical studies of the Phlebotomus fever group viruses.

Analyses of the virion polypeptides and genomic RNA species of the Phlebotomus fever group viruses (Bishop, et al., 1978; Robeson et al., 1979; Bishop, 1979; Bishop and Shope, 1979; Bishop et al., 1980) have been continued and are directed towards determining whether these viruses structurally parallel other member of the Bunyaviridae by having tripartite genomes and three major virion polyepeptides. Since serologic evidence has indicated that there is no detectable serologic homology between members of the Bunyavirus genus members and the Phlebotomus fever group viruses (see (6) below), we have been interested in determining if a rationale can be developed for forming a new genus of bunyaviruses based on the unique structural and serologic properties of the Phlebotomus fever group members. The results obtained have justified the formation of the Phlebovirus genus of the Bunyaviridae. This genus has been proposed to the International Committee on the Taxonomy of viruses and accepted (Bishop et al., 1980).

The structural analyses have been undertaken as the prelude to determining if recombination involving RNA segment reassortment occurs among phleboviruses and which viral RNA species codes for which virion polypeptide.

a. RNA analyses.

The information developed in prior years on the virion RNA sizes of KAR, PT, ITP, CDU, CHG and, in part, on SFS and SFN viruses has been extended to include the Tesh and Sabin isolates of SFS, Buenaventura (BUE) and Icoraci (ICO) viruses. For each virus three RNA species (L, large, M, medium, S, small) have been identified and from coelectropherograms of their viral RNA species with the viral RNA species of snowshoe hare (SSH) virus, the apparent molecular weights have been determined for PT, KAR, CHG, SFN and both SFS derivatives. In summary, the data reveal that the RNA species of the viruses have mol. wt. values of 2.6-2.8x10^6(L), 1.85-2.2x10^6 (M) and 0.7-0.8x10^6(S). The L and M RNA mol. wt. values resemble those of Bunyavirus genus members (Bishop and Shope, 1979). The S RNA is significantly larger than the S RNA of Bunyavirus genus members (average 0.44x10^6, Ushijima et al., 1980).

Composite and individual L, M and S RNA fingerprints (Clewley and Bishop, 1980; Clewley et al., 1977a,b) were reported previously for KAR and PT viruses (Robeson et al., 1979). Similar data has been obtained in the current reporting period for SFS (Tesh), SFS (Sabin) and BUE viruses. The results indicate that each virus has RNA species with distinguishable fingerprints and unique L, M and S RNA sequences.

b. Viral polypeptides.

In last year's report we demonstrated that the G1 and G2 polypeptides of KAR behave aberrantly in polyacrylamide gels. For reduced virus samples one glycopolypeptide band is obtained (Robeson et al., 1979). For unreduced samples two glycopolypeptides are recovered (Robeson et al., 1979) and it was shown by tryptic peptide analyses that these two glycopolypeptides have unique sequences (Cash et al., 1980). We also reported previously that the KAR G1 and G2 polypeptides form the surface spikes seen in electron micrographs of purified virus preparations (Robeson et al., 1979).

We have extended the analyses of the virion polypeptides of KAR virus to PT and CHG viruses with the results shown in Fig. 1. These results indicate that the N polypeptides of PT and CHG viruses have similar mol. wts. (24x10^3), slightly larger than that of KAR virus (21x10^3). Two glycopolypeptides were seen in the preparation of PT virus (68x10^3, 60x10^3), one in CHG virus with a mol. wt. of 58x10^3 as well as another approximately twice the size of the 58x10^3 dalton polypeptide. The glycopolypeptides of CHG virus were also found to behave aberrantly in polyacrylamide gel analyses. For fresh virus preparations a single polypeptide band is observed at 58x10^3 daltons. On storage, a larger mol. wt. species (as seen Fig. 1) appears and does not disappear by boiling, or storing the viral polypeptides in the presence of up to 5% DTT or mercaptoethanol. This larger species may be a dimer of the smaller species.

Similar large dimers of KAR viral glycopolypeptides have been observed by rerunning purified ^{3}H-methionine labeled KAR G1 with ^{35}S-methionine labeled KAR G2 polypeptide, each originally purified as single bands with molecular weights of 62x10^3 and 50x10^3 respectively (Fig. 2).

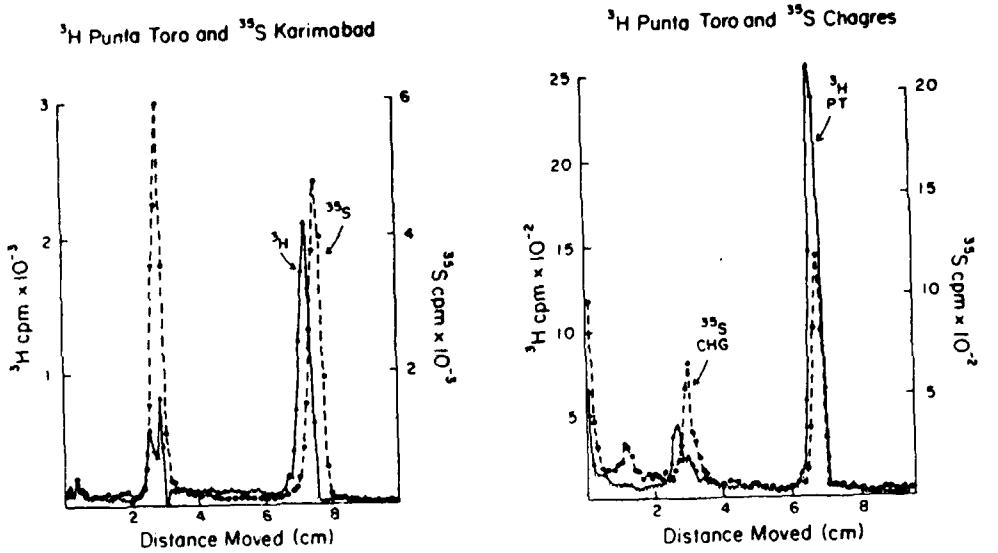


Figure 1. Coelectrophoreses of the viral polypeptides of ^{3}H-methionine labeled PT virus with (left panel) ^{35}S-methionine labeled KAR virus and (right panel) ^{35}S-methionine labeled CHG virus.

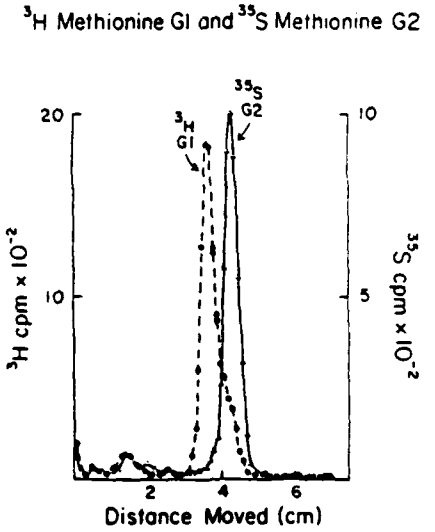


Figure 2. Coelectrophoresis of gel purified ^{3}H-methionine labeled KAR G1 with gel purified ^{35}S-methionine labeled KAR G2 polypeptide.

The identification of the viral glycopolypeptides of PT, CHG and other members of the Phlebotomus fever serogroup has been achieved by the use of dual labels (e.g. ^{3}H-glucosamine and ^{35}S-methionine) as shown in the results presented in Figure 3 for PT virus.

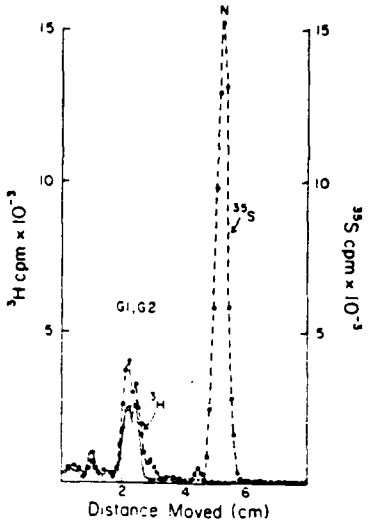


Figure 3. Polyacrylamide gel electrophoresis of the virion polypeptides of (^{3}H)glucosamine and (^{35}S)methionine labeled PT virus.

The results of almost all the polypeptide analyses that have been performed so far are shown in Table 4. Phl 3 virus is a recent sandfly fever virus isolated from Italy, it is reportedly serologically related to SFN. The results indicate that the viruses have a non-glycosylated $20\text{-}24\times10^{3}$ polypeptide (N), and glycosylated polypeptides of mol. wt. $57\text{-}69\times10^{3}$. Whether virions have minor quantities of other polypeptides is not known, nor have the relationships of the viral glycopolypeptides been proven for all the viruses shown. Finally we have noted that the degree of separation obtained between G1 and G2 polypeptides depends on the gel system employed (phosphate or Tris-glycine) as well as in some cases (e.g. KAR) the presence or absence of a reducing agent.

Table 4. Mol. wt. estimations of the virion polypeptides of phleboviruses

Virus	Glycopolypeptides (G1, G2)	Nucleocapsid protein (N)
*KAR	62×10^{3}	21×10^{3}
SFS(Tesh)	69×10^{3}	24×10^{3}
SFS(Sabin)	69×10^{3}, 66×10^{3}(?)***	24×10^{3}
SFN	67×10^{3}, 57×10^{3}	22×10^{3}
**CHG	58×10^{3}	24×10^{3}
PT	68×10^{3}, 60×10^{3}	24×10^{3}
ICO	62×10^{3}	20×10^{3}
Phl 3	67×10^{3}, 62×10^{3}	22×10^{3}
BUE	60×10^{3}, 58×10^{3}	21×10^{3}

*Single bands are seen in reduced virus preparations, two bands in unreduced preparations.

**Fresh virus preparations yield one polypeptide band; virus preparations that have been stored with or without DTT or mercaptoethanol always give a dimer glycopolypeptide band.

***We are not certain that the smaller mol. wt. band is viral in origin.

c. Surface structure.

Studies by Saikku, von Bonsdorff, Oker-Blom and associates on Uukuniemi (UUK) virus (von Bonsdorff et al., 1969; von Bonsdorff and Pettersson, 1975; Pettersson et al., 1971; Saikku and von Bonsdorff 1968; Saikku et al., 1970) indicate that although this tick-borne virus resembles the bunyaviruses in general morphological properties, hexagonal arrays of surface components can be visualized, particularly after glutaraldehyde fixation. Such arrays, although occasionally seen for other viruses, have not been seen when La Crosse virus is treated similarly (Obijeski et al., 1976; F. Murphy, personal communication). The reason is not known. Since the glycoprotein species of UUK virus (75 and 65×10^3 daltons) are quite different to those of La Crosse virus (110 and 38×10^3 daltons), it is possible that the surface arrangement of UUK virus reflects these differences. The clustered projections observed for UUK virus are in the form of hollow cylindrical morphological units, 8-10 nm long and 10-12 nm in diameter, with a central 5 nm cavity (von Bonsdorff and Pettersson, 1975). Both negative staining and glutaraldehyde fixation plus freeze-etching of UUK virus preparations have suggested that the surface units are penton-hexon clusters arranged in a T=12, P=3 icosahedral surface lattice with hexon-hexon distances estimated to be between 12.5 and 16 nm for stained virus particles and 17 nm for the freeze-etched samples. The analyses performed on UUK virus preparations also indicate that the surface subunits are probably attached to a lipid bilayer which can be freeze-fractured.

Since the glycoprotein sizes of the Phlebotomus fever serogroup viruses (vide infra) more closely resemble those of UUK virus than bunyaviruses (von Bonsdorff and Pettersson, 1975; Pettersson et al., 1971, 1977), we have sought to determine the surface structure arrangement of the virus particles of several members of the serogroup. Shown in Figure 4 are the electron micrographs of pelleted preparations of SFS, CHG and ICO viruses, as well as an electron micrograph of a thin section of PT virus infected cells. Each of the virus preparations was obtained from cell supernatant fluids that were initially clarified by centrifugation at 8,000xg ($4^{\circ}C$) and incubated in the presence of glutaraldehyde (1% final concentration) for 60 min ($18^{\circ}C$) then pelleted through a cushion of 30% glycerol. As the higher magnification of SFS (x204,000) reveals, a clustering of the surface components could be detected on the virus particles. The PT infected cell shows virus particles in intracellular vacuoles.

When KAR virus particles were purified by glycerol-tartrate gradient centrifugation (and dialysis to remove the salts), then treated with sodium phosphotungstate and examined in the electron microscope, mostly spherical virus particles were revealed with an electron lucent envelope to which were attached an external fringe of surface components (Fig. 5.1, magnification x287,000). Shown in Fig. 5.4 (magnification x75,000) is a KAR virus particle which was penetrated by the stain (see arrow). When PT virus was similarly analysed by negative staining (but not treated by glutaraldehyde) deformed particles were frequently observed (Fig. 5.3, magnification x106,400). In Fig. 5.5 are shown two KAR virus particles pretreated with 1% glutaraldehyde (magnification x170,000) in which the surface arrangement is more evident than in the untreated particles (Fig. 5.1). Also shown in Fig. 5 (Fig. 5.3, magnification x186,000) are negatively stained KAR virus particles pretreated with chymotrypsin - which evidently removed the surface spikes and glycoprotein (Robeson et al., 1979)

Figure 4

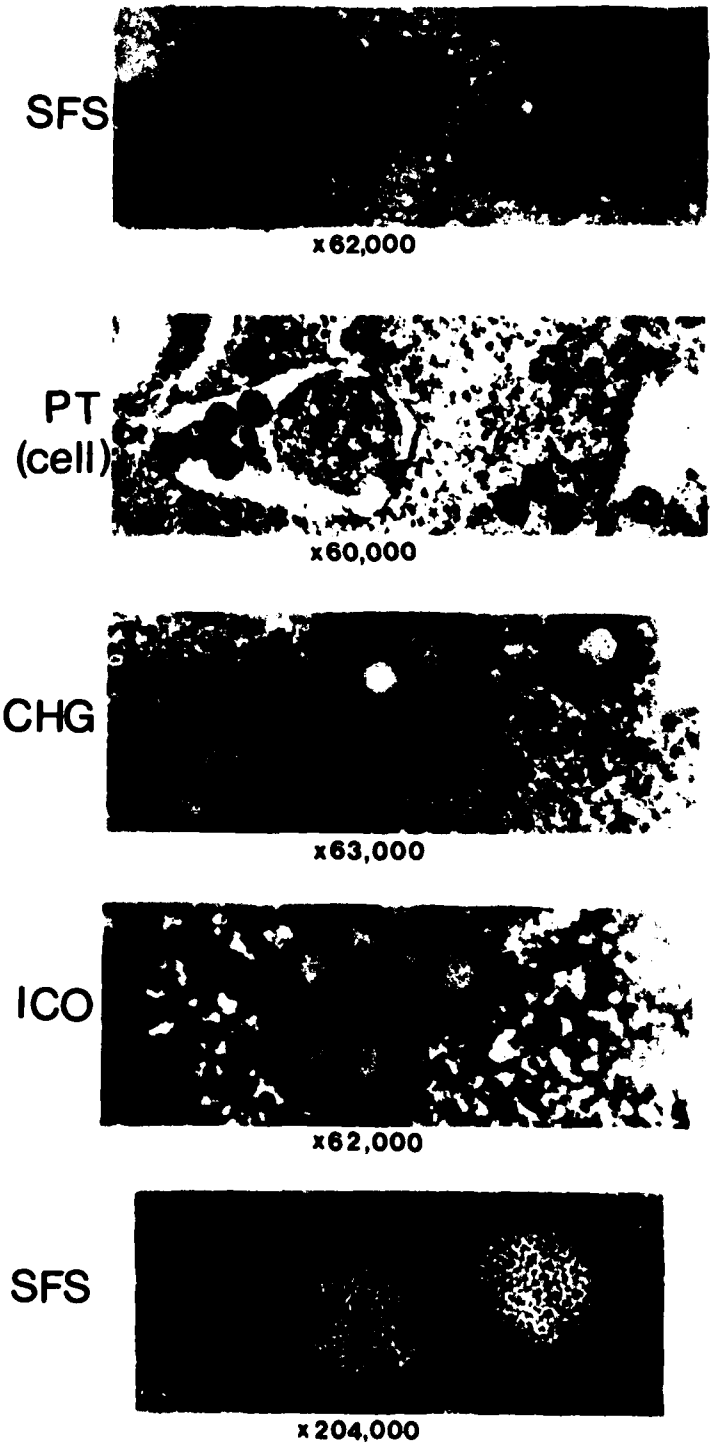
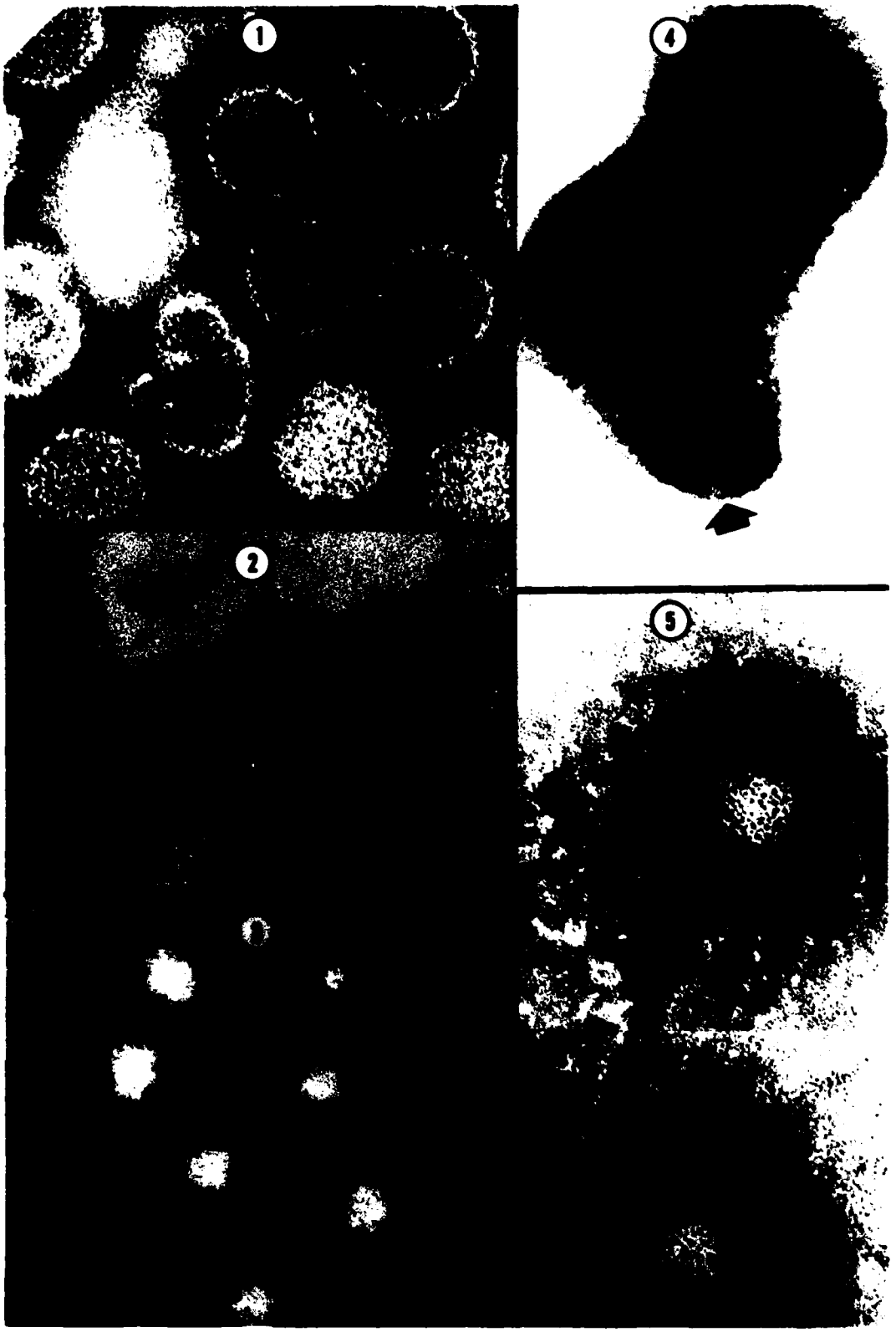


Figure 5



In summary, structural analyses of certain of the Phlebotomus fever group viruses have established that they have three unique viral RNA species and at least for KAR (and probably other members of the group) three major polypeptides (G1, G2 and N). These analyses confirm the structural similarities to the Bunyavirus genus members. However, by contrast to all the Bunyavirus genus members so far analyzed (Obijeski and Murphy, 1977; Bishop and Shope, 1979; Ushijima et al., 1980), whereas the latter have a large 110-120x10^3 dalton G1 and a smaller 32-38x10^3 dalton G2 polypeptide, the Phlebotomus fever group viruses have two glycoproteins of similar size (52-69x10^3 daltons) arranged (like the Uukuniemi viruses) in a particular surface arrangement. All three virus groups (bunyaviruses, Uukuniemi and Phlebotomus fever serogroup viruses) have a similar 20-25x10^3 dalton nucleocapsid protein, N.

(6). Competition RIA analyses with KAR viral polypeptides.

We have begun an analysis of the antigenic determinants of phleboviruses in order to determine if correlations can be made between antigenic similarities and genetic abilities. Although PT virus would be the best basis for comparison (in view of the genetic analyses we have undertaken), we have instead chosen to start with KAR virus because we can obtain adequate quantities of purified virus for iodination experiments. PT virus has been very difficult to obtain with enough glycoprotein for iodination (see Fig. 3) although we intend to pursue purification procedures which yield enough intact PT virus for such analyses. The preliminary studies we report here have therefore used KAR N and glycoproteins as bases for comparison.

Hyperimmune antisera was raised in a rabbit by intraperitoneal and intradermal injection of a total of 300 µg of purified virus particles, at intervals of two weeks. Subsequent to a prebleed, the first injection consisted of 150 µg of virus mixed with an equal volume of Freund's complete adjuvant (total volume 1.0 ml). Two weeks later a booster of 150 µg of virus mixed with an equal volume of Freund's incomplete adjuvant (total volume 1.0 ml) was injected. After an additional 2 weeks 50-100 ml of blood were collected and serum prepared from the whole blood after a 30 min incubation at 37°C followed by an overnight incubation at 4°C. Cells were removed by centrifugation at 2500 x g for 15 min.

The method of virus fractionation after detergent and high salt treatment is essentially that reported by Obijeski et al., (1976). Purified virus was harvested from a 70-20% sucrose gradient and pelleted through a one ml glycerol pad by centrifugation in a Beckman SW41 rotor at 40,000 rpm for two hr at 4°C. The pellet was taken up in 5 ml of solution containing 1.0 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.002 M EDTA and 2% (wt/vol) Triton X-100. This mixture was incubated at 37°C for 30 min and then centrifuged in a Beckman SW50.1 rotor at 45,000 rpm for two hr at 4°C. The viral glycoproteins were recovered from the resulting supernatant by ethanol precipitation, and the nucleocapsid pellet washed with 0.01 M Tris-HCl, pH 7.4, and finally resuspended in a small volume (0.1 ml) of 0.01 M Tris-HCl, pH 7.4. Quantitation of the protein in the final suspension was performed using a Bio-Rad protein assay kit (Bio-Rad, La Jolla, California).

Purified viral protein preparations were labeled with (^{125}I)sodium iodide using either the chloramine T or lactoperoxidase methods (Greenwood et al., 1963; Murphy, 1976). Viral polypeptides (10-25 µg aliquots) suspended in 25-50 µl of 0.01 M sodium phosphate buffer, pH 7.2, were labeled in vitro by chloramine T by incubating the protein sample for four min at room temperature with 10 µl of 2.5 mg

per ml aqueous solution of chloramine T. Radiochemical oxidation of the polypeptides was minimized by terminating these chloramine T reactions after the four min incubation. Lactoperoxidase catalysed iodination involved incubating a protein sample (10-25 µg) at room temperature for 20 min with 50 µl of the bead-attached enzyme. After incubation the beads were removed by centrifugation in the Beckman J-6B centrifuge at 2,000 rpm for five min. Immediately following radioiodination, the products were separated from unbound (^{125}I)sodium iodide by passage through a Bio-gel P-2 column (BioRad, Richmond, Calif.) equilibrated with RIAB (0.1% Triton X-100, 0.02 M Tris-HCl, pH 7.6, 0.1 M NaCl, 0.001 M EDTA, fraction V bovine serum albumin at 10 mg/ml). The purity and integrity of all polypeptide preparations were verified by polyacrylamide gel electrophoresis. The specific activities of the iodinated viral preparations were determined and only samples with a specific activity above 20,000 cpm/ng protein were used for RIA determinations. The purities of the iodinated polypeptides were verified by immunoprecipitation. Viral polypeptide samples (10 µl) were mixed with 5 µl of undiluted hyperimmune antisera raised against the homologous virus and 200 µl of a 10% (wt/wt) suspension of A-protein bearing Staphylococcus aureus (Cowan strain I) added (Kessler, 1975). After 30 min incubation at 37°C, the mixture was pelleted and washed 2 times with cold RIAB. The final pellet was taken up in TDB (0.0625 M Tris-HCl pH 6.8, 5% SDS, 1% mercaptoethanol, 10% glycerol, 0.005% bromophenol blue), resuspended by sonication, boiled for three min, to dissociate the immune complexes from the bacteria, then repelleted to remove the bacteria. The resulting supernatant containing the liberated polypeptides was recovered and resolved by polyacrylamide gel electrophoresis.

The procedure for competitive radioimmunoassay was essentially that described by Hunter et al., (1978). An antiserum was initially titered against a labeled antigen preparation both to determine the maximal amount of antigen that could be precipitated from the preparation (usually 10-50% of the total cpm), and to determine the dilution of serum that would give 50% of the maximal precipitation. One hundred µl of that serum dilution was mixed with 25 µl of the labeled antigen (50,000-180,000 of the precipitable cpm) and 25 µl of unlabeled competing antigen. The competing antigen was used at a variety of ten-fold serial dilutions made in RIAB, starting with an initial concentration of 10-20 mg per ml. The mixtures were incubated at 37°C for 30 min, at which time 100 µl of the 10% (wt/wt) Staph A solution was added. The antigen-antibody-bacterium-complex was incubated at 4°C for 30 min and then pelleted 5,200 rpm and 4°C in a Beckman J-6B centrifuge. The resultant pellet was washed 3 times with RIAB and counted. In all cases the competing cold antigen was purified whole virus that had been treated with 1% Triton X-100 and incubated at 37°C for 30 min with 25 µg/ml RNase A. When plotting the results, the % maximal precipitation was taken to be the number of counts precipitated in the absence of competing cold antigen minus the number of counts precipitated by a prebled normal rabbit serum control (usually 4-10% of the total precipitable cpm). All experiments were performed in duplicate.

Figure 6 presents the results of a homologous competitive RIA with ^{125}I-labeled KAR glycopolypeptides, KAR antiserum raised in a rabbit and the unlabeled competing viral antigens of the phleboviruses KAR, SFS and CHG, those of the bunyaviruses LAC, ORI and BUN, and the viral antigens of the unrelated rhabdovirus, VSV. Two sources of LAC viral antigens were used, those from virus grown in BHK-21 cells and those obtained from LAC virus grown in Vero cells, the cells that are used for growing phleboviruses (Robeson et al., 1979). This latter

source of LAC virus was employed in order to determine if the host cell used to prepare virus had an effect on the competitions.

KAR viral antigens exhibited a typical homologous competition curve against the KAR labeled glycopolypeptides. SFS viral antigens also competed well for the KAR antibodies, while CHG virus did not compete as well (Fig. 6). Neither the viral antigens of VSV, nor those of the selected bunyaviruses (LAC, ORI or BUN), competed for the KAR antibodies reacting with the labeled KAR glycopolypeptides. No competition by the viral antigens of LAC virus grown in Vero cells was detected, indicating that the assay apparently does not measure any host specified antigenic determinants on LAC virus preparations.

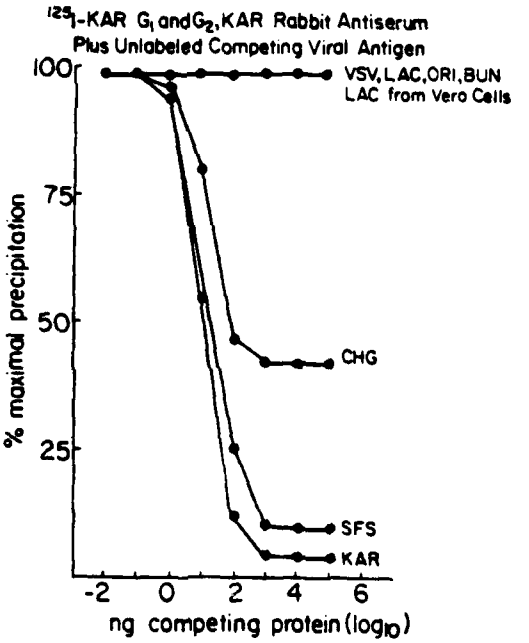


Figure 6. An explanation of the 0-100% maximal precipitation is presented in the text. In this experiment the 0-100% range was approximately 50×10^3 cpm. The KAR glycoprotein antigens were prepared by the Triton X-100 and 1 M NaCl procedure and were labeled by the lactoperoxidase method.

Figure 7 presents the results of a homologous competitive RIA with ^{125}I-labeled KAR N polypeptide, KAR antiserum raised in a rabbit and the unlabeled competing viral antigens of the phleboviruses KAR, SFS and CHG, as well as those of the bunyaviruses LAC, ORI and BUN, and the viral antigens of the unrelated rhabdovirus, VSV. Again two sources of LAC viral antigens were used, those from virus grown in BHK-21 cells and those obtained from LAC virus grown in Vero cells.

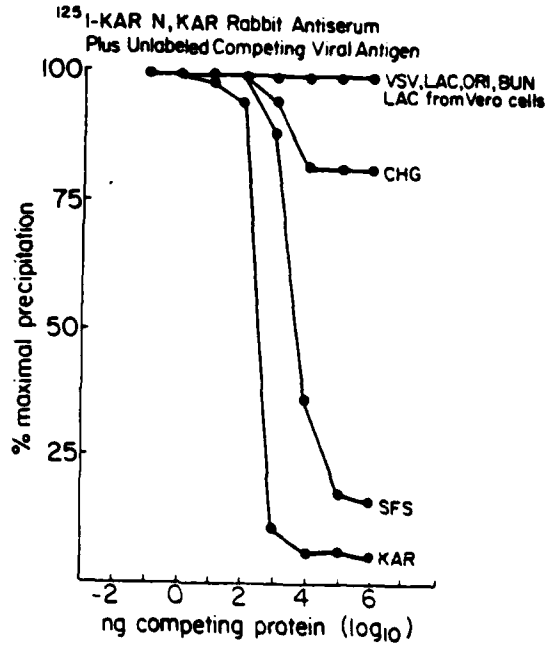


Figure 7. An explanation of the 0-100% range is presented in the text. In this experiment the 0-100% range was approximately $3x10^3$ cpm. The KAR N antigen was generated by pelleting KAR nucleocapsids after release from virions by the Triton X-100 and 1 M NaCl procedure. The N polypeptide was labeled by the chloramine T method after digestion of the nucleocapsids by ribonuclease.

As seen in the previous experiment, members of the Bunyavirus genus, or the unrelated virus, VSV, failed to compete for antibodies reacting with the antigenic determinants recognized on the KAR N polypeptide. The SFS viral antigens gave competition plateau levels like those obtained with KAR N protein, although the viral antigens of CHG virus were poor competitors against the labeled KAR N polypeptide, poorer than the equivalent experiment involving CHG glycoproteins (Fig. 6). This result indicates that the antigenic determinants on the CHG glycoproteins are more conserved than those of its N protein. Whether this a situation unique to CHG virus, or is shared by other members of the serogroup remains to be determined. While it may indicate greater conservation of antigenic determinants on one viral protein than on another, a consideration that should be borne in mind is that if the two antigens are coded by different viral RNA segments, as in the bunyaviruses, then the differences could reflect the results of ancestral reassortment of the viral genome RNA segments.

7. Genetic analyses with other phleboviruses.

We have initiated genetic studies with other members of the Phlebotomus fever serogroup (phleboviruses) by attempting to adapt the various viruses to growth and plaquing at 39.8°C. The viruses currently under study are KAR, BUE (CoAr 3319, the nearest serologic relative of PT virus), ICO and SFS (Sabin). None of the original stocks of these viruses gave plaques at 39.8°C although all gave plaques at 33°C, 35°C and 38°C. Although we could use 38°C as the nonpermissive temperature we prefer to use 39.8°C since 4.8°C (39.8-35°C) is a better range for differentiating ts viruses than 3°C. The plaques at 33°C are pinpoint, and contain less than 10^3 PFU per plaque, making screening for ts mutants (with 33°C as the permissive temperature), a laborious process. We could use 33°C as the permissive temperature and 38°C as the nonpermissive temperature, but at the present time we elect to use 35°C as the permissive temperature and 39.8°C as the nonpermissive temperature.

The results of passaging virus at 39.8°C to select for high temperature adapted virus are promising for ICO virus. The initial two passages at 39.8°C gave virus stocks with titers of 1.1×10^5 and 9.5×10^4 PFU/ml (at 35°C) and no plaques at 39.8°C. The third passage gave 8.4×10^4 PFU/ml at 35°C and 2.3×10^3 PFU/ml at 39.8°C. Currently we are recloning the virus by selecting the largest plaques (from this stock) at 39.8°C and will grow new virus stocks to obtain high titered virus. We hope by this means to obtain a virus stock that gives plaques at 39.8°C with reasonable EOP values that we can use for genetic analyses. Further passaging at 39.8°C and recloning may be needed to increase the EOP.

The results of high and low temperature passaging of BUE virus have been hampered by problems encountered in maintaining virus titers. Each passage at 35°C has resulted in a log drop of virus yields and no plaques have been obtained after three 39.8°C passages. We suspect that defective interfering virus may be causing this problem and we are currently recloning the virus to generate new stocks to work with.

So far we have only passaged SFS (Sabin) and CHG twice at 39.8°C, and have no results to report on their adaptation. Further passaging and recloning is needed.

8. Fingerprint analyses of RVF virus isolates.

Rift Valley fever (RVF) virus is a newly assigned virus to the Phlebovirus genus based on data developed by USAMRIID and YARU personnel (Shope et al., 1980). Studies on the structural componants of RVF (Rice et al., 1980) indicate that RVF virus has 3 major polypeptides (G1 65×10^3, G2 55×10^3, N 25×10^3) and 3 RNA species (2.7×10^6 L, 1.7×10^6 S) like other members of the Phlebovirus genus.

The RVF viral RNA segments, labelled with ^{32}P-orthophosphate, have been separated by sedimentation on a sucrose gradient and the L and M RNA species recovered and digested with ribonuclease T1. The digests were resolved by 2-dimensional gel electrophoresis and the oligonucleotide fingerprints obtained (Fig. 8). Unfortunately the recovery of the viral S RNA segment was insufficient to analyse by fingerprinting. Also shown in Figure 8 is the RNA fingerprint of the total viral RNA, (i.e. prior to fractionation on a sucrose gradient). It was concluded from the results obtained that, like other Phlebotomus fever viruses, the L and M RNA

segments contain unique sequences (see the arrows in the individual and composite fingerprints in Fig. 8).

Figure 8. Ribonuclease T1 oligonucleotide fingerprints of RVF ZZ 501 L RNA (left hand panel), M RNA (center panel), and total viral RNA (right hand panel). The oligonucleotide fingerprints were obtained as described by Clewley and associates (1977). The arrows indicate unique oligonucleotides. Insufficient RVF S RNA was obtained for a similar analysis.

The extent of variation of the genomes of RVF isolates has been determined by comparing of the composite RNA fingerprints of six isolates of RVF. The place and date of origin of the isolates we have used were as follows: (1) Entebbe, Uganda (1944); (2) Kimberley, South Africa (1951), sheep isolate; (3) Salisbury, Rhodesia (1970), human isolate; (4) Sinoia, Rhodesia (1974); Zagazig, Egypt, (1977), human isolate; (6) Asyut, Egypt (1978), bovine isolate. All of these isolates have been identified as RVF by serology.

Figure 9 shows a comparison of the composite RNA fingerprints of the two Egyptian isolates, ZZ 501 (1977), and ZC 3349 (1978). The location of the unique L and M RNA oligonucleotides previously identified for ZZ 501 (Fig. 8) are indicated by arrows. No differences in the large unique oligonucleotides (lower half of the fingerprint) were found between these two isolates, suggesting that a single strain of RVF circulated in Egypt in 1977 and 1978. A greater degree of diversity was observed for the two Rhodesian isolates (separated in time by four years) as shown in the bottom two panels of Fig. 10. Likewise both the South African and Uganda isolates were quite distinct by comparison to each other (Fig. 10), or to the Rhodesian or Egyptian isolates. Thus despite the fact that all these isolates are serologically identical, they can be distinguished by their RNA sequences (Cash et al., 1980).

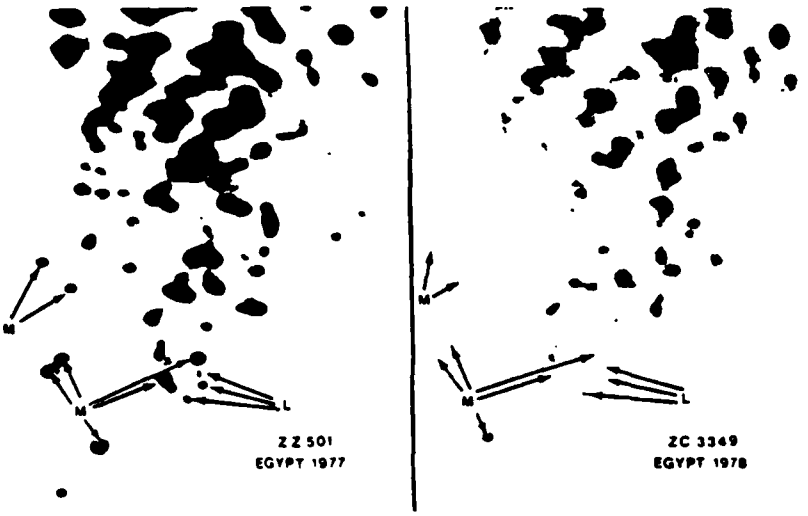


Figure 9. Ribonuclease T1 oligonucleotide fingerprints of the Egyptian RVF isolates: ZZ 501 and ZC 3349. The composite (L/M/S) oligonucleotide fingerprints of two strains of RVF ZZ 501 (Egypt, 1977) and ZC 3349 (Egypt, 1978) were obtained as described by Clewley and associates (1977). The arrows in the two panels indicate unique oligonucleotides of the L and M RNA segments (see Fig. 8).

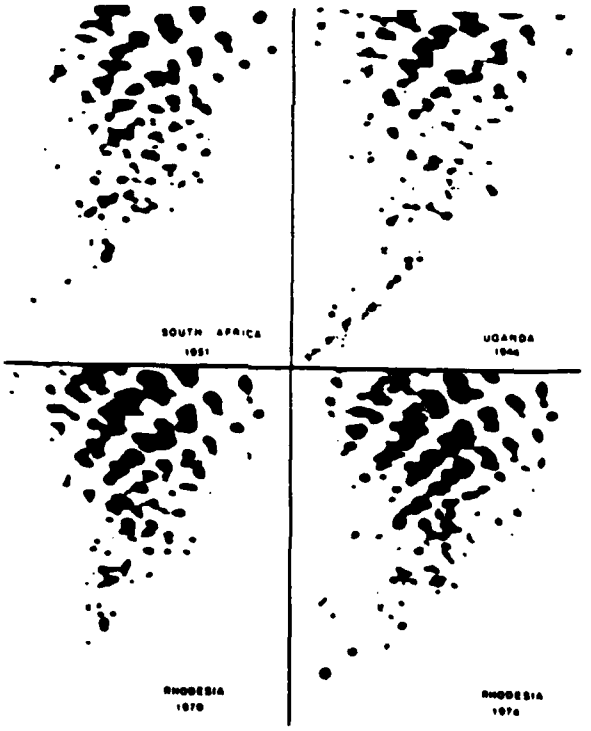


Figure 10. Ribonuclease T1 oligonucleotide fingerprints of four RVF isolates: South Africa (1951); Uganda (1944); Rhodesia (1970); Rhodesia (1974). The composite (L/M/S) oligonucleotide fingerprints of four strains of RVF were obtained as described by Clewley and associates (1977). Although there appear to be some similarities between the fingerprints of the four isolates overall the results indicate that they have unique RNA sequences, further analyses are required to determine the degree of relatedness.

The oligonucleotide fingerprint analyses we have preformed here demonstrated that RVF has undergone evolutionary changes over the years, and that a number of distinct virus varieties are circulating in nature. The relationships of the various strains to each other are not known, nor can we determine from the limited analyses we have conducted so far, where the Egyptian virus came from. However as more strains are analysed, and the number of circulating varieties determined, it should be possible to determine the relationships of the various virus isolates to each and from where the Egyptian isolates originated.

B. Summary of Progress Report.

The research supported by this contract has shown that representative Phlebotomus fever group viruses have a tripartite RNA genome consisting of 3 unique RNA species (L, M and S). The L and M RNA species have mol. wts. like those of Bunyavirus genus members. However their S RNA species is significantly larger than that of Bunyavirus genus members. Like members of the Bunyavirus genus, the Phlebotomus fever serogroup virus have a major 20-24x10^3 dalton nucleocapsid protein N and, at least for KAR virus (and probably other members of the serogroup), two external, unique, glycoproteins G1 and G2. The analyses have shown that the size ranges of the glycoproteins of the Phlebotomus fever group viruses are quite different to those of members of the Bunyavirus genus. The glycoproteins of several members of the group are in a surface arrangement like the glycoproteins of Uukuniemi virus, but unlike those of Bunyavirus genus viruses. Rift Valley fever virus also appears to have viral RNA species and polypeptide sizes like those of the Phlebotomus fever group viruses and unlike those of the Bunyavirus genus (Rice et al., 1980; Cash et al., 1980). Various RVF virus isolates have been analyzed by oligonucleotide fingerprinting with results that indicate the genetic variation that exists for RVF on the African continent.

The biochemical data developed from the research supported by this contract have been the base for the recent proposal (and acceptance) of the group as a new genus in the Bunyaviridae, named the Phlebovirus genus.

Genetic studies initiated with Punta Toro (PT) virus have shown that high frequency genetic recombination occurs during certain mixed virus infections. Temperature sensitive (ts) mutants of PT virus have been categorized into groups on the basis of their recombination capabilities (Group I has 8 ts mutants; Group II has 5 ts mutants; Group III has 1 ts mutant; 1 ts mutant is unassigned). Initial experiments on high temperature adaptation are being undertaken on BUE, KAR, SFS, and ICO viruses, prior to seeking ts mutants of these viruses. When ts mutants are obtained they will be used in recombination experiments with PT ts mutants to determine the genetic capabilities of the viruses and the RNA coding assignments.

Radioimmune assays have been developed with KAR virus to eventually relate the genetic capabilities of the phleboviruses with their antigenic similarities. The preliminary results indicate that SFS and KAR share more common N and glycoprotein antigenic determinants than KAR and CHG viruses.

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